Yeasts in foods and beverages: in vitro characterisation of probiotic traits

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Abstract

The possible beneficial properties of food and associated microorganisms for both human and animal health are increasingly investigated. While many bacteria have been characterised as probiotics, only one yeast variety, *Saccharomyces cerevisiae* var. *boulardii*, has been recognised to be part of this group, regardless of the importance of yeasts in the food industry. In this work, we have studied the probiotic or health-beneficial potential of 130 yeast strains isolated from food belonging to the collection of the Institute of Food Science Research CIAL (CSIC-UAM). To that end, the response of these yeasts to physiological conditions during consumption has been assessed through measuring their capacity to grow at 37 °C and to survive the gastrointestinal transit conditions. Almost 50% of the yeast analysed could thrive at the host intestine temperature, and of these, almost 37% had growth kinetic parameters higher at 37 °C than at 25 °C. In addition, about 95% of the strains could survive the exposition to conditions simulating the gastrointestinal transit. The yeast strains were also analysed for exhibiting auto-aggregation phenotype, antioxidant activity and the production of killer peptides, this only found in 8.5% of the strains analysed. These properties could be regarded as additional interesting features for selecting new probiotic strains.

Keywords: yeasts, gastrointestinal transit, probiotics, antioxidant activity, killer peptides.

1. Introduction

Yeasts are a big and heterogeneous group of eukaryotic microorganisms belonging to the phyla Ascomycetes and Basidiomycetes, widely distributed and used in biotechnology for different purposes, such as production of fermented foods, alcoholic fermentation, recombinant protein and vitamin synthesis and biological control (García-Hernández et al., 2012; Hatoum et al., 2012). They are of great interest to the food industry, since they can be used as starters in the production of various foods and beverages such as bread, wine, beer, kefir, koumiss, or table olives (Arroyo-López et al., 2012; Moreira et al., 2011), and participate in the maturation of some cheeses (Binetti et al., 2013). Yeasts are also commonly present in numerous foods (e.g. beer, wine, bread, fruit juices, mayonnaise or chocolate) as contaminants that can cause food spoilage (Lowes et al., 2000). Nevertheless, many yeast species have been proven to have antimicrobial activity against spoilage microorganisms or pathogens (Antunes and Aguiar, 2012; Lowes et al., 2000).

Probiotics are defined as live microorganisms which, administered in adequate amounts, confer a beneficial effect on the host health (FAO/WHO, 2006). Several criteria are required for a microorganism to be considered as probiotic: it must be innocuous, causing neither toxicity nor illness to the host, it must be in high amounts in the product with which it is administered and maintain its viability during its shelf-life, it must be technologically usable and survive the gastrointestinal transit (de Vrese and Schrezenmeir, 2008; Tripathi and Giri, 2014; Hill et al., 2014). There is a new trend in food microbiology in the use of multifunctional microorganisms like starter cultures with probiotic activity (Perricone et al., 2014); therefore, it is interesting to evaluate additional functional properties in yeasts tested for probiotic traits, such as antioxidant

activity (Chen et al., 2010), inhibition of pathogens (Binetti et al., 2013; Silva et al., 2011) and killer phenotype (Silva et al., 2011).

Multiple evidence point that many pathological processes (e.g. rheumatoid arthritis, osteoarthritis, cardiovascular disease and some tumours) are secondary to an oxidative damage produced by free radicals, especially reactive oxygen species (ROS) (Kawagishi and Finkel 2014; De Rosa et al., 2010; Valko et al., 2006). A reduction of this oxidative damage can lower the risk of developing these diseases (Halliwell, 2011, Serafini, 2006); hence there is an increasing interest in the study of natural antioxidants. It has been recently reported that yeasts and yeast extracts have antioxidant activity due to a wide range of substances (e.g. superoxide dismutase, catalase, oxygenated carotenoids, resveratrol, octacosanol, etc.), amongst which the (1→3)-β-D-glucan and other β-glucans found in the cell wall have an essential role (Abbas, 2006; Jaehrig et al., 2007).

Inhibition of other yeasts by mycocinogenic strains is mainly mediated by the production of killer peptides or mycocins, to which the producer strain is immune (Golubev, 2006; Marquina et al., 2002). Mycocins are proteins or glycoproteins between 10 and 30 kDa, conventionally constituted by two or three subunits (Golubev, 2006), which have been found to be widely spread amongst yeasts (Lowes et al., 2000).

The majority of probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium* (Hill et al. 2014; de Vrese and Schrezenmeir 2008), and regardless of their importance and wide distribution in foods, the probiotic or beneficial potential of yeasts is usually overlooked (Chen et al., 2010). To this day, *Saccharomyces cerevisiae* var. *boulardii* is the only yeast recognised and characterised as probiotic (Czerucka and Rampal 2002; Hatoum et al., 2012), but this raises the question whether other yeast

strains (specially *S. cerevisiae*) possess probiotic properties as well (Binetti et al. 2013; van der Aa Kühle et al., 2005).

Thus, the aim of the present work was to evaluate the probiotic potential of 130 yeast strains belonging to the microbial culture collection of the Institute of Food Science Research CIAL (UAM-CSIC), which had been mostly isolated from wine or grape must. Particularly, the capacity of the strains to grow at 37 °C and the survival to the gastrointestinal (GI) transit were assessed, as well as the antioxidant activity, and the killer and auto-aggregation phenotypes.

2. Materials and methods

2.1. Strains: origin and culture conditions

In this work, 130 yeast strains belonging to the CIAL (CSIC-UAM) collection were studied. From all the strains, 129 (99.2% of the total) were isolated from different food products and beverages (66 strains from grape must), while only one strain (*Candida inconspicua* IFI-1328) was isolated from a non-alimentary source (compost). Regarding their geographic origin, 127 strains were isolated from various regions of Spain, while only three strains came from Italy. The studied strains are encompassed in 25 different species (Table 1), included in the genera *Candida* (2 strains), *Kluyveromyces* (4 strains), *Lachancea* (1 strain), *Metschnikowia* (5 strains), *Pichia* (10 strains), *Saccharomyces* (68 strains), *Schizosaccharomyces* (5 strains), *Torulaspora* (23 strains), *Wickerhamomyces* (9 strains), *Yarrowia* (2 strains) and *Zygosaccharomyces* (1 strain). These strains had previously been identified by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers, as described by Esteve-Zarzoso et al. (1999).

The yeasts were preserved in YPD (yeast extract 10 g/L, bacteriological peptone 20 g/L, dextrose 20 g/L) agar (20 g/L) slants at 4 °C. For their use, each strain was

transferred into YPD agar plates and incubated at 25 °C for 3 – 7 days. Before each assay, the strains were grown in YPD broth at 25 °C for 24 h.

2.2. Growth at 37 °C and survival to simulated GI digestion

An overnight culture incubated at 25 °C of each strain was inoculated at 1% in fresh YPD broth, dispensed in duplicates in 96-well plates and incubated in an automated microplate spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA), taking measurements of the OD₆₀₀ at regular time intervals during 72 h. Each strain was incubated at 25 and 37 °C, thus allowing us to compare the kinetic growth parameters at both temperatures. The data were obtained with the software *SkanIt software 2.4.1* and treated with the application *DMFit* (Institute of Food Research, United Kingdom) for Excel 2010 (Microsoft Corporation) to obtain the growth curves and parameters (lag time and maximum growth rate $\mu_{\max}$). Those strains capable of growing at 37 °C were further analysed to check their survival to simulated GI digestion. For this purpose, they were exposed to two models of GI transit, each one consisting of two consecutive steps simulating the conditions in stomach and intestine and differentiated by the presence of pepsin.

– Model A: each strain was harvested by centrifugation (12000 rpm, 5 min) and washed twice with sterile NaCl solution (0.9%). Cells were resuspended in YPD broth pH 3 (adjusted with 6 M HCl) and incubated at 37 °C during 2 h, the exposure time to stomach acidic conditions recommended by Minekus et al. (2014). Afterwards, cells were harvested (12000 rpm, 5 min), washed twice and resuspended in YPD broth with 3.0 g/L bile salts (Fluka Chemie AG, Buchs, Switzerland). The samples were then incubated 3 h at 37 °C.

– Model B: the strains were harvested (12000 rpm, 5 min), washed twice and resuspended in sterile NaCl solution (0.5%) with 3.0 g/L porcine pepsin (Sigma-

Aldrich, St Louis, MO, USA) and pH adjusted to 3 with 6 M HCl. After incubation at 37 °C during 2 h, cells were harvested (12000 rpm, 5 min), washed twice and resuspended in sterile NaCl solution (0.5%) with 3.0 g/L bile salts and pH adjusted to 8 with NaOH (1 M). Afterwards, the tubes were incubated 3 h at 37 °C.

After exposition to each model of GI digestion, survival of each strain was tested by inoculating fresh YPD broth at 1% with the bile salt-treated yeast suspension and measuring the OD₆₀₀ every hour during 72 h of incubation at 37 °C in the automated microplate spectrophotometer. The data were obtained and treated as described above to compare the damaging effect of each GI digestion model. We also tested the capacity of the strains to grow in YPD broth supplemented with 0.3% bile salts after exposition to model A. To achieve this, each treated strain was inoculated in fresh YPD broth at 1% with 0.3% bile salts and incubated at 37 °C for 72 h in the automated microplate spectrophotometer. The OD₆₀₀ was measured every hour.

2.3. Antioxidant activity

To evaluate the antioxidant activity, the percentage of reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was performed with the protocol described by Chen et al. (2010) with some variations. Briefly, 1 mL of yeast culture in YPD broth was harvested by centrifugation (12000 rpm, 5 min), washed twice with a sterile solution 0.9% NaCl and the resulting pellet resuspended in 1 mL of the same solution. The cell suspension (800 µL) was transferred into a new tube, where 1 mL of a DPPH solution (0.2 mM in methanol) was afterwards added. The mix was vortexed and then incubated 30 min at room temperature in darkness. The reaction tubes were centrifuged (12000 rpm, 5 min) and 300 µL of the supernatant were transferred into 96-well plates in order to measure the absorbance at 517 nm (A₅₁₇). The percentage of reduction of DPPH was calculated as follows: $[1 - A_{517(\text{sample})} / A_{517(\text{blank})}] \times 100\%$

We confirmed experimentally that the percentage of scavenging of DPPH is directly proportional to the OD₆₀₀ of the cell solution used. Hence, we measured the initial OD₆₀₀ of each culture and standardised the results for an OD₆₀₀ = 1.20. We also assessed the Trolox equivalent antioxidant capacity of each strain using a solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in methanol (1 mM).

2.4. Antimicrobial activity. Killer phenotype

The killer phenotype assay was performed according to the method used by Llorente et al (1997) with some modifications. This characteristic was tested in YPD agar plates adding different NaCl concentrations (0%, 4% and 6%) and adjusting the pH (pH values 6.35 [not adjusted], 4.8 and 4) with 200 mM phosphate-citrate buffer (19.2 g/L citric acid, 14.2 g/L Na₂HPO₄). The resulting media used were YPD agar with no supplements, YPD agar 4% NaCl (pH not adjusted), YPD agar 6% NaCl (pH not adjusted), YPD agar 4% NaCl pH 4.8, YPD agar 6% NaCl pH 4.8, YPD agar 6% NaCl pH 4. The plates were seeded with a killer-sensitive control strain (*S. cerevisiae* BY-4741) using sterile cotton buds. Once dried, 4 µL of each yeast grown in YPD broth were inoculated in spots on the top of the sensitive strain, and the plates were incubated at 20 and 25 °C during 5 – 7 days. Each assay was performed in duplicates.

As the killer phenotype is usually exhibited against strains of the same species or taxonomically near species (Golubev, 2006), we also tested those strains belonging to species different from *S. cerevisiae* (4 strains of *Schizosaccharomyces pombe*, 3 strains of *Saccharomyces bayanus* and 3 strains of *Wickerhamomyces* spp) against strains from the same species. The procedure was the same, in this case seeding the agar plates with these strains instead of with the killer-sensitive control strain *S. cerevisiae* BY-4741.

2.5. Auto-aggregation

To measure the auto-aggregation percentage, 1.2 mL of a yeast culture in YPD broth was harvested by centrifugation (12000 rpm, 5 min) and washed twice with a sterile solution 0.9% NaCl. The resulting pellet was resuspended in 1 mL of the same solution and transferred to a disposable plastic cuvette. Without altering the microbial suspension, the OD₆₀₀ was measured at 0, 2, 4 and 24 h in a Specord 2010 plus spectrophotometer (Analytik Jena, Germany). The auto-aggregation percentage was calculated as follows: $[1 - (A_T/A_0)] \times 100\%$

Where A_T is the OD₆₀₀ at each assay time and A_0 is the OD₆₀₀ at inoculation time.

2.6. Statistical analysis

Percentages, mean values, medians and standard deviations were calculated based on the values for the different variables studied. Determination of statistically significant differences ($P < 0.05$) were performed by t analysis for mean values of two paired samples. All the calculations were carried out with the software *Data analysis* in Excel 2010 (Microsoft Corporation).

3. Results and discussion

3.1. Growth at 37 °C and survival to simulated GI digestion

Out of the 130 strains analysed, 62 (47.7%) were able to grow at 37 °C (Table 2). Five *S. cerevisiae* strains (IFI-81, IFI-92, IFI-130, IFI-244 and CYC-SGA) did not present statistically significant differences in their $\mu_{\max}$ at 25 and 37 °C, suggesting a similar growth capacity at both temperatures, and 21 strains (16.2% of the total) had a $\mu_{\max}$ significantly higher ($P < 0.05$) at 37 °C than at 25 °C. For two strains (*M. pulcherrima* IFI-1242 at 37 °C and *C. inconspicua* IFI-1328 at 25 °C), the latency time (lag) value could not be obtained, and only 15 strains (11.5% of the total) presented shorter lag at 37 °C than at 25 °C (*S. cerevisiae* IFI-87, IFI-132, IFI-242, IFI-506, IFI-715, IFI-722, IFI-1145, BY-4741 and CYC-SGA, *S. pombe* IFI-356, IFI-936 and IFI-

1148, *W. subpelliculosa* IFI-920, *P. membranaefaciens* IFI-946 and *W. anomalus* IFI-1378).

Overall, many of the yeast strains tested could grow at 37 °C, a desired condition for a microorganism to be tested for probiotic traits. Furthermore, the strains *P. membranaefaciens* IFI-946, *S. cerevisiae* IFI-1145 and *S. pombe* IFI-356 and IFI-1148 showed both $\mu_{\max}$ and lag values higher and shorter at 37 °C, respectively, thus indicating that 37 °C is a more favourable temperature for their propagation and, thereby, that they can thrive at the host temperature, which could support their viability instead of acting as a stress factor. This is interesting when selecting new potential probiotic strains, since LAB usually show a high trend towards surviving to higher temperatures than yeasts (Romero-Gil et al., 2013).

The tolerance to the GI transit of the 62 strains able to grow at 37 °C was afterwards tested. After exposition to model A (evaluation of tolerance to acidic conditions and bile salts) 59 strains (95.2%) presented growth in YPD broth, while only three strains (*S. exiguus* IFI-658, *S. cerevisiae* IFI-715 and *K. waltii* IFI-1147) showed no growth. Furthermore, 40 of these 59 strains could grow in YPD broth supplemented with 0.3% bile salts (*P. membranaefaciens* IFI-946 was the only strain that presented poor growth), suggesting an overall high resistance of yeast strains to the GI transit stress. In all strains except five (*S. pombe* IFI-356, *T. delbrueckii* IFI-746, *M. pulcherrima* IFI-1242, *W. subpelliculosa* IFI-1253, and *C. inconspicua* IFI-1328), the $\mu_{\max}$ was significantly lower when cultivated in YPD broth supplemented with 0.3% bile salts than when grown in YPD broth (data not shown). The results obtained after exposition to model B (evaluation of tolerance to acidic conditions, pepsin and bile salts) in terms of number of strains capable of surviving were similar. Of the 62 strains tested, 57 were able to grow after exposition to model B of GI transit, and only two

strains (*S. cerevisiae* IFI-242 and IFI-465) survived the exposition to model A but not to model B (Table 2). The $\mu_{\max}$ mean value obtained for the yeast strains exposed to model A was 0.12 ± 0.05 (median 0.13), while this value for model B was 0.06 ± 0.05 (median 0.05), significantly lower. Similarly, the lag mean value obtained for model A was significantly shorter ($8.15 \text{ h} \pm 4.59$, median 7.69) than that obtained for model B ($21.75 \text{ h} \pm 8.98$, median 18.70), thus indicating that pepsin may influence negatively the yeast survival to GI stress conditions both prolonging the lag phase and hindering the growth. On the other hand, two *S. cerevisiae* strains (IFI-87 and IFI-132) presented lag values longer after exposition to model A than to model B.

Within the different yeast species analysed, of the 59 strains that survived the exposition to the GI transit, 45 (76.3%) were *S. cerevisiae*, while only 14 strains (23.7%) belonged to other species encompassed in the genera *Saccharomyces*, *Pichia*, *Schizosaccharomyces*, *Torulaspora*, *Metschnikowia*, *Wickerhamomyces* and *Candida*. These results suggest that different yeast genera, and though specially, not only the genus *Saccharomyces*, can harbour strains with probiotic potential. Some strains from these non-*Saccharomyces* species have been assayed as potential probiotics for animal production (Gil de los Santos et al., 2012; Gatesoupe, 2007).

3.2. Antioxidant activity

All the strains tested exhibited antioxidant activity in different levels (Table 3). Among the 59 yeasts analysed, only one strain (1.7%) showed low antioxidant activity, with DPPH scavenging percentage under 20%, six strains (10.2% of the total) presented an activity between 20 and 30%, 36 strains (61.0%) showed a good activity, between 30 and 40%, 14 strains (23.7%) exhibited very good activity (between 40 and 50%) and two strains (3.4%) displayed excellent activity with a percentage of reduction of DPPH exceeding 50% (Table 3). The lowest value was obtained for *S. cerevisiae* IFI-88 (19.74

$\pm 0.12\%$), and the highest for *S. pombe* IFI-936 ($50.72 \pm 0.16\%$) and *S. cerevisiae* IFI-279 ($56.97 \pm 0.30\%$). The antioxidant activity was also measured as trolox equivalents (TE, μM), obtaining an activity range between 73.39 ± 0.73 TE (for *S. cerevisiae* IFI-88) and 177.60 ± 0.85 (for *S. cerevisiae* IFI-279). These results are similar to those found by Chen et al. (2010) when analysing intact yeast cells, with a percentage of antioxidant activity ranging from 4.25 to 46.78%. The authors obtained lower antioxidant activities with yeast extracts, correlating with the results obtained by Naylin et al. (2005). The yeast antioxidant activity is believed to be mainly due to the high content of (1 $\rightarrow$ 3)- β -D-glucan and other β -glucans found in the cell wall (Abbas, 2006; Jaehrig et al., 2007), and other cellular compounds, such as some antioxidant enzymes like superoxide dismutase, glutathione peroxidase and catalase (Chen et al., 2010), but as intact cells show conventionally higher activities than cell extracts, other mechanisms may be involved. On the other hand, the antioxidant activity of yeasts seems higher than that of lactic acid bacteria (LAB). In our study, the antioxidant activity in TE was generally higher than that reported by Amaretti et al., (2013) for different LAB strains, with a maximum mean value of 125 TE obtained for *Lactobacillus brevis* DSMZ 23.

3.3. Antimicrobial activity. Killer phenotype

The killer phenotype assay was tested in YPD agar plates adding different NaCl concentrations and varying the pH and the temperature of incubation. When a positive result was yielded, the halos obtained were generally bigger when the incubation temperature was 20 °C than when it was 25 °C, and in some cases, killer phenotype was exhibited at the first temperature, but not at the latter, confirming the importance of temperature in the killer phenotype assay (Golubev, 2006; Hernández et al., 2008). The medium in which the bigger inhibition halos were registered was YPDA with 6% NaCl and pH adjusted to 4, with the highest NaCl concentration, in accordance with Llorente

289 et al. (1997), and under the most acidic conditions, as stated by Golubev (2006). Only *S.*
 290 *bayanus* IFI-702, *S. cerevisiae* IFI-716 and CYC-SGA, *M. pulcherrima* IFI-1242 and
 291 *W. subpelliculosa* IFI-1253 (8.5% of the 59 strains studied) displayed killer activity in at
 292 least one of the conditions tested. The first three strains revealed their killer phenotype
 293 against several strains in different conditions, while the last two were only active
 294 against the killer sensitive control strain, *S. cerevisiae* BY-4741 and only when the
 295 assay medium was YPD with 6% NaCl and pH 4, at both 20 and 25 °C. *S. bayanus* IFI-
 296 702 showed the broadest spectrum of killer activity. This strain was active against the
 297 killer sensitive control strain, *S. cerevisiae* BY-4741, *S. pombe* IFI-356, IFI-1184 and
 298 IFI-2180 and *S. bayanus* IFI-697 and IFI-704. Furthermore, it displayed its killer
 299 activity in most of the conditions tested, i.e. YPDA 6% NaCl at both temperatures,
 300 YPDA 4% NaCl pH 4.8 at 20 °C, YPDA 6% NaCl at pH 4.8 and pH 4 at both
 301 temperatures. *S. bayanus* IFI-702 was even active, though weakly, against several
 302 strains when the culture media used were YPD 4% NaCl (against *S. bayanus* IFI-704
 303 and *S. pombe* IFI-1184) and YPD without supplementation (against *S. cerevisiae* BY-
 304 4741 and IFI-1148 and *S. bayanus* IFI-697 and IFI-704). *S. cerevisiae* IFI-716 exhibited
 305 the second broader spectrum, being active against the same strains than *S. bayanus* IFI-
 306 702 in almost the same assay conditions. When the test culture medium was YPD
 307 without supplementation, this strain was just weakly active against *S. pombe* IFI-1148.
 308 The killer positive control strain, *S. cerevisiae* CYC-SGA, had a narrower but still broad
 309 spectrum of inhibitory activity. Its killer phenotype was highlighted against less strains
 310 (*S. cerevisiae* BY-4741, *S. pombe* IFI-356 and *S. bayanus* IFI-697 and IFI-704) and in
 311 less culture conditions (YPDA 6% NaCl at 20 °C, YPDA 4% NaCl pH 4.8 at 20 °C,
 312 YPDA 6% NaCl pH 4.8 at both temperatures and YPDA 6% NaCl pH 4 at both

temperatures) than the aforementioned strains. This strain did not show any killer phenotype neither in YPD 4% NaCl nor in YPD without supplementation.

Overall, the killer phenotype was not widely distributed amongst the yeasts tested, as it only appeared in 8.5% of the strains. Interestingly, all the killer positive yeasts exhibited activity against strains belonging to other species, and sometimes, even to different genera. We have found crossed activity between the species *S. bayanus*, *S. cerevisiae* and *S. pombe*, of two taxonomically related genera, but we have also reported inhibitory activity between more distantly related genera: two strains from the genera *Metschnikowia* and *Wickerhamomyces* inhibited the growth of a strain of the genus *Saccharomyces*. The results regarding the narrow spectrum of *M. pulcherrima* IFI-1242 contrast with those found by Oro et al. (2014), who reported inhibitory activity of seven *M. pulcherrima* strains against several yeast genera, but not against *S. cerevisiae*. Killer phenotype is an interesting technological characteristic especially considered in wine-making, to avoid contaminations by wild yeasts, and in biocontrol, to preserve stored fruits, cereals (Antunes and Aguiar 2012) or brined olives against spoilage (Hernández et al., 2008).

3.4. Auto-aggregation

Other of the desirable characteristics of a potential probiotic microorganism is the ability to form cellular aggregates, since aggregates can increase the microbial adherence to the intestine, thus providing advantages in the colonization of the GI tract (García-Cayuela et al., 2014). Yeast cells are bigger and heavier than bacteria, and therefore, they precipitate quicker and in higher proportion. In accordance, all the auto-aggregation percentages obtained were high at 24 h (Fig. 1), ranging from $83.3 \pm 0.1\%$ for *S. cerevisiae* IFI-87 to $99.8 \pm 0.0\%$ for *S. pombe* IFI-936 and *S. cerevisiae* IFI-244. At 2 h, the auto-aggregation percentages showed a high level of variability, oscillating

from $1.1 \pm 0.0\%$ for *S. cerevisiae* IFI-460 to $85.8 \pm 0.2\%$ for *T. delbrueckii* IFI-746, suggesting that auto-aggregation percentages are strongly strain dependent. All the strains displayed a fast auto-aggregation, occurring within the first 4 h of incubation, and only one strain, *M. pulcherrima* IFI-1242, has an auto-aggregation percentage higher from 4 to 24 h than from inoculation time to 4 h. As can be seen in Fig. 1, for each strain the higher aggregation percentages usually occurred between 2 and 4 h (in 44 out of 59 strains, 74.6%), with values between 23.4% (*T. delbrueckii* IFI-746) and 89.1% (*S. cerevisiae* IFI-244). In the remaining 25.4% of the strains (15 out of 59: *S. cerevisiae* IFI-87, IFI-88, IFI-463, IFI-465, IFI-692, IFI-716, IFI-722, and CYC-SGA; *S. bayanus* IFI-702 and IFI-704; *T. delbrueckii* IFI-746; *P. membranaefaciens* IFI-946; *S. pombe* IFI-1148 and IFI-2180; and *W. anomalus* IFI-1378), the percentage of aggregation was higher between inoculation time and 2 h. Our auto-aggregation results at 2 h were similar, but with a wider range, than those obtained by Binetti et al. (2013), who reported values from $16 \pm 0.1\%$ to $70 \pm 2.5\%$ at the same time of incubation. It may be due to the higher number of strains analysed in our work.

Auto-aggregation or most commonly called in yeasts, flocculation, is a phenomenon that occurs often upon sugar depletion during late exponential or stationary phase (Goossens and Willaert 2010). This process depends on several aspects, such as cell-surface physicochemical properties, presence of Ca^{2+} or mannose in the medium (Touhami et al., 2003), presence of any of the *FLO* genes encoding lectin-like proteins known as adhesins, zymoclectins or flocculins (van Mulders et al., 2010), or the culture replicative age (Powell et al., 2003). Auto-aggregation is mediated by cell-surface molecules, and therefore, it is affected by the different cell-wall composition of each strain and the presence of appendages or macromolecules

protruding from the wall (Touhami et al., 2003). This may explain the differences found in our study among the strains tested.

4. Conclusions

Adaptation to 37 °C, resistance to the GIT stress conditions and auto-aggregation phenotype are general features observed in the wine-related yeast strains analysed in this study. Within the observed properties, the antioxidant activity and killer peptide phenotype found in several strains belonging to non-*Saccharomyces cerevisiae* species could be regarded as additional interesting features for selecting new probiotic strains. In this regard, *S. pombe* IFI-936 and IFI-2180 have displayed a high capacity to thrive in the host intestine (better growth capacity at 37 °C than at 25 °C, good tolerance to GIT stress conditions, and high autoaggregation percentage at early test time) and a high antioxidant activity. The results obtained in this study require further studies about the health benefits of different yeast species and the mechanisms involved, as well as the safety assessment of non-*Saccharomyces* strains.

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